**Pioglitazone up-regulates MALAT1 and promotes the proliferation of endothelial progenitor cells by increasing c-Myc expression in type 2 diabetes mellitus**

Running title: The role of pioglitazone in EPC function

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**Abstract**

**Objective:** Evidence suggests that pioglitazone improves the function of endothelial progenitor cells (EPCs). However, the knowledge of its molecular mechanism remains limited. In the present study, the role of long non-coding RNA MATAL1 in modulating the number and function of EPCs in type 2 diabetes mellitus (T2DM) was characterized. **Methods**: Circulating EPCs were obtained from peripheral blood of healthy subjects and T2DM patients with or without pioglitazone treatment. The *in vitro* experiments were conducted in bone marrow EPCs to evaluate the effect of pioglitazone and its possible mechanism. **Results**: Pioglitazone increased circulating EPCs number and improved their function in T2DM patients. Transfected EPCs with siRNA-MALAT1, the increase of c-Myc protein expression induced by pioglitazone treatment was canceled. In db/db diabetic mice, a glucose tolerance test showed that pioglitazone increased glucose tolerance, which was reversed by siRNA-MALAT1. **Conclusion**: Pioglitazone improves EPCs function possibly through regulating MALAT1 and c-Myc expression in T2DM.

**Keywords:** pioglitazone; diabetes mellitus; MALAT1; endothelial function; glucose tolerance

**Introduction**

Pioglitazone, the peroxisome proliferator-activated receptor-gamma (PPARγ) agonist, is an insulin-sensitizing agent acting as a prescription drug for the treatment of type 2 diabetes mellitus (T2DM) [1, 2]. It has been demonstrated that pioglitazone provides benefits in a diverse population of patients with diabetes, with lowering the risk of death, myocardial infarction, or stroke [3]. A subset of stem cells named endothelial progenitor cells (EPCs) exist with the vascular circulation and play important roles in the maintenance of endothelial homeostasis and vascular integrity, contributing to vessel repair following endothelial damage [4]. Reduced EPCs number and function are associated with the presence of cardiovascular events including T2DM [5-7]. Pioglitazone involves controlling blood glucose through insulin-sensitizing effects [8]. In addition, the emerging evidence indicates that the improvement of endothelium-dependent vascular function also participates in the protective effect of pioglitazone on T2DM and its associated complications [9]. In the last decade, the role of pioglitazone in EPCs has been highlighted. Spigoni, et al showed that pioglitazone treatment improves *in vitro* viability and function of EPCs and reduces cardiovascular risk in impaired glucose tolerance individuals independent of the insulin-sensitizing action [10], and PI3K/Akt signal pathway has been proposed in this process [11]. However, the knowledge remains limited in the molecular mechanisms of pioglitazone on EPCs regulation.

Non-coding RNAs have been widely considered to take part in various pathophysiologic processes of animal and human diseases [12, 13]. Long non-coding RNA (lncRNA), has more than 200 nucleotides, is one type of the novel non-coding RNAs and participates in the modulation of gene transcription, post-transcriptional regulation and epigenetic regulation [14, 15]. Metastasis associated lung adenocarcinoma transcript 1 (MALAT 1), also known as noncoding nuclear-enriched abundant transcript 2 (NEAT2), is first found in lung cancer and regulates the expression of metastasis-associated genes [16, 17]. The newly published paper showed that MALAT1 controls a phenotypic switch in endothelial cell and regulates its function and vessel growth [18]. It also has been demonstrated that MALAT1 plays a pathogenic role in endothelial cell dysfunction in diabetes mellitus [19]. However, whether MALAT1 is involved in regulating EPCs function in T2DM remains unknown. The objective of this study was to examine the effect of pioglitazone on EPCs function and to evaluate whether MALAT1 participates in this process.

**Materials and Methods**

**Subjects**

The human study was designed as a prospective clinical trial on healthy subjects and patients with T2DM. All participants were recruited from Tianjin Medical University Chu Hisen-I Memorial Hospital. The trial was performed with the approval of the Ethics Committee of Tianjin Medical University Chu Hisen-I Memorial Hospital. Informed consent was obtained from all participants.

Thirty-two healthy subjects (control) and 65 T2DM patients were recruited. Thirty-two patients with T2DM received the treatment of pioglitazone (30 mg/day) for four weeks, and the other 33 patients received no treatment. In this study, the T2DM was defined according to the American Diabetes Association criteria [20]. Patient with one or more than one of the following criteria were excluded: type 1 diabetes, treatment with insulin, treatment with diet and/or exercise, treatment with any thiazolidinedione, treatment with medications known to affect EPC biology. After four weeks after treatment, fasting venous blood was collected. The fresh whole blood was used to assess the number and function of circulating EPCs. The serum was obtained to perform the routine examination.

**Flow cytometry analysis of EPCs number**

Hematopoietic stem cells (CD34 and CD133) and endothelial cells (KDR) are the surface markers of EPCs. The determination of these three markers represents the number of EPCs. Flow cytometry was used to determine the expression of CD34, CD133 and KDR of mononuclear cells (MNCs). A volume of 10 mL fresh peripheral venous blood with the treatment of heparin (20 u/mL) was used to obtained MNCs using Ficoll-Hypaque Solution (Tianjin Hanyang Biologicals Technology Co., Ltd, China). MNCs were then re-suspended into M199 medium (Hyclone, USA) and diluted in FACS buffer for the measurement of CD34, CD133 and KDR with incubating with anti-CD34-PC-5 (Becton Dickinson, USA), anti-CD133-FITC (Beijing Bo Orson Biological Technology Co., Ltd., China) and anti-KDR-PE (R&D Systems, USA) in the dark. Cells were washed twice with FACS buffer and fixed with 2% paraformaldehyde. The analysis was performed by assessment of 100, 000 events in each separate examination. The expression of CD34, CD133 and KDR was reported as a percentage of total events.

**EPCs** **adhesion**

Adherent cells were dissociated with 0.25% trypsin and re-suspended in medium. The cell suspension was washed with PBS for 5 min and seeded at 1×105 cells in fibronectin-coated culture plates for 30 min. Cells were counted under a light microscope.

**Migration assay**

Boyden chambers were used to assess the migration of EPCs. Treated the Boyden chamber (Haimen Qi LinBeiEr instrument manufacturing co., LTD., China) with ultraviolet irradiation overnight. Cells were dissociated with trypsin (200 μL/well) and re-suspended in M199 medium with 5% fetal bovine serum. The concentration of cells was adjusted to 2.0×105 cells/well. The lower chamber was added with M199 medium containing 20 μL vascular endothelial growth factor (VEGF, 50ng/mL). A cell suspension of 200 μL was placed in the upper chamber with polycarbonate-free membrane (8.0 μm proes) to culture for 90 min. Cells in the upper chamber were removed and the migrated cells on the lower surface of the membrane were stained with haematoxylin and eosin and counted under the microscope.

***In vitro* angiogenesis assays**

Angiogenic capacity of EPCs was measured by the*in vitro* Angiogenesis Assay Kit (Chemicon, USA) according to manufacturer’s instruction. Cells were harvested with trypsin, and cultured in a 96-well plate pre-coated with ECMatrix gel for 24h. The cells were counted under the microscope and tube formation was calculated.

**Bone marrow-EPC culture**

*In vitro* experiments were performed using bone marrow-EPCs culture. Healthy male C57BL/6 mice aged 8–10 weeks were sacrificed after anesthetization with ketamine (25 mg/kg). Femurs and tibias were dissected, the adhering tissues were completely removed and both ends of the bones were excised. Bone marrow cells (BMCs) were harvested by flushing with Endothelial Cell Growth Medium 2 (EGM-2, Lonza, USA) and density gradient centrifugation method. Cells were re-suspended in EGM-2 containing 20% fetal calf serum (FCS), and seeded at a concentration of 1×106 cells/mL in culture plates at 37°C with 5% CO2. Adherent cells were incubated with fresh EGM-2 at day 3 after removing unattached cells. The medium was replaced every 2 days. The morphology of cells was monitored. When the cells reached 80% confluence (at day 7 to 10), 0.125% trypsin was used for cell dissociation.

**RNA extraction and quantitative real-time PCR**

The circulating EPCs or bone marrow-EPCs were lysed by TRIzol reagent (Invitrogen, USA) and total RNA was extracted according to the manufacturer’s instruction. PrimeScriptTM RT-PCR Kit (Takara, China) was used to synthesize the cDNA with RNA. For the quantitative analysis of MALAT1 and c-Myc, the BIO-RAD CFX96 touch q-PCR system was applied with IQ SYBR Green supermix (Bio-Rad Laboratories, USA). All samples were read in triplicate, and the relative expression values were normalized to GAPDH and β-actin expression with 2−ΔΔCT method, respectively.

**Western blot**

For western blot analysis, cells were harvested, washed twice in cold PBS. Then they were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitor for 1 h. Lysates were centrifuged at 10, 000×g for 10 min at 4°C, and supernatants collected. The protein concentration was measured by Bradford Protein Assay Kit (Beyotime, China). Equal amount of protein extract of each sample was loaded onto 10% SDS-PAGE electrophoresis for separation and then transferred to PVDF membranes (Millipore, USA) with blocking in 5% nonfat milk prior to incubation with the primary antibodies against c-Myc and β-actin (1:1000, Cell Signaling Technology, USA) overnight at 4 °C. The membrane was then incubated with HRP-conjugated secondary antibody (1:5000, Cell Signaling Technology, USA) for 90 min.

**Animal experiments**

C57BL/6J db/db diabetic mice at ten weeks of age were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All animals were fed with a standard chow diet in a temperature-controlled room. Isolated autologous EPCs from mice were used to treat diabetic mice. Before the injection, EPCs were pre-incubated with 10 mM Pioglitazone for 2h and transfected with pLVX-IRES-ZsGreenl-si-MALAT1 (pLVX-IRES-ZsGreenl as control) for 24h. EPCs were intravenously injected into the mice once a week for 16 weeks. A glucose tolerance test was performed to determine glucose tolerance. In brief, after fasting for 15 hours, mice received 0.5g/kg glucose by intraperitoneal injection. Blood was collected from the tail vein and the blood glucose concentration measured. The animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and approved by the animal experimental ethics committee of Tianjin Medical University Chu Hisen-I Memorial Hospital.

**Statistical analysis**

All statistical analyses were performed using SPSS 13.0 statistic software. Data were represented as mean ± standard error (SEM). The difference of means between two groups was analyzed by Student's t test. A P value less than 0.05 was regarded as statistically significant.

**Results**

**Pioglitazone improves the function of circulating EPCs in T2DM patients.**

The number and function of circulating EPCs in T2DM patients were first evaluated. As expected, T2DM patients had decreased EPCs markers CD34+, CD34+KDR+ and CD34+KDR+CD133+ numbers compared with healthy subjects (**Figure 1A**). The function of circulating EPCs was then measured. The results showed that all migration, adhesion and formation of EPCs were reduced to 43.4%, 50.9% and 58.1% in T2DM patients, respectively (**Figure 1B-1D**). The number and function of circulating EPCs in T2DM were significantly elevated in patients given 30 mg pioglitazone for 4 weeks.

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**Figure 1.** **Effects of pioglitazone on the number and functions of circulating EPCs in T2DM patients.** The EPCs number (A), adhesion (B), migration (C) and tube formation (D) were detected in healthy subjects or T2DM patients with or without the treatment of pioglitazone (30 mg pd for four weeks). \*\* *P*<0.01, vs. control; ## *P*<0.01, vs. diabetes.

**MALAT1 and c-Myc expression of circulating EPCs in T2DM patients**

The MALAT1 level of circulating EPCs was then investigated in T2DM patients, and the result showed that it was significantly down-regulated to 38.3%, whereas pioglitazone treatment resulted in an increase of MALAT1 expression level to 81.3% (**Figure 2A**). c-Myc has been demonstrated to play a vital role for adequate angiogenesis and regulating endothelial dysfunction [21, 22]. The c-Myc mRNA and protein expression levels of circulating EPCs in T2DM patients presented a similar trend with respect to MALAT1, while pioglitazone treatment up-regulated these expressions (**Figure 2B**).

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**Figure 2. Effects of pioglitazone on MALAT1 and c-Myc expression of circulating EPCs in T2DM patients.** The expression levels of MALAT1 RNA (A) and c-Myc mRNA and protein (B) were determined in healthy subjects or T2DM patients with or without treatment of pioglitazone (30 mg pd for four weeks). \*\* *P*<0.01, vs. control; ## *P*<0.01, vs. diabetes.

**Pioglitazone affected the MALAT1 and c-Myc expression in bone marrow-EPCs exposed to high glucose**

The effects of pioglitazone in the *in vitro* model of T2DM were demonstrated. Co-incubated bone marrow-EPCs with different concentrations of glucose (1%, 2%, 5% and 10%), showed a declining MALAT1 and c-Myc mRNA and protein expression in a dose-dependent manner (**Figure 3A** and **3B**). However, the decrease of MALAT1 and c-Myc expression in EPCs exposed to high glucose (10%) could also be reversed by treatment with pioglitazone *in vitro* (**Figure 3C** and **3D**).

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**Figure 3. Effects of pioglitazone on MALAT1 and c-Myc expression in bone marrow-EPCs exposed to high glucose *in vitro*.** The expression levels of MALAT1 RNA (A) and c-Myc mRNA and protein (B) were determined in bone marrow-EPCs with different concentrations of glucose (1%, 1%, 2%, 5% and 10%). These expressions (C, D) were also determined in high glucose (10%) induced bone marrow-EPCs with the treatment of pioglitazone. \*\* *P*<0.01, vs. control; ## *P*<0.01, vs. diabetes.

**MALAT1 down-regulation affected the effect of pioglitazone on c-Myc expression**

Pioglitazone has been confirmed to play a role in reversing the effect of high glucose on MALAT1 and c-Myc expression in bone marrow-EPCs. Thus when bone marrow-EPCs were administrated si-MALAT1 for down-regulating its expression, the role of pioglitazone in increasing c-Myc protein expression was largely canceled (**Figure 4A**). However, the mRNA expression of c-Myc was not changed by si-MALAT1 (**Figure 4B**).

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**Figure 4. MALAT1 is involved in the effect of pioglitazone on c-Myc expression in bone marrow-EPCs exposed to high glucose *in vitro*.** The expressions of c-Myc in protein level (A) and in mRNA level (B) were determined in bone marrow-EPCs with different treatments. \*\* *P*<0.01, vs. NG; ## *P*<0.01, vs. NG+DMSO; $$ *P*<0.01, vs. NG+DMSO+si-control.

**MALAT1 down-regulation affected c-Myc expression and stability**

In order to confirm the role of MALAT1 in regulating c-Myc expression, bone marrow-EPCs were co-incubated with si-MALAT1 alone to observe c-Myc expression. As shown in **Figure 5**, c-Myc protein expression was decreased to 51.0% (**Figure 5A**) but its mRNA expression was not altered (**Figure 5B**) by si-MALAT1. In addition, c-Myc protein expression was decreased with the time of si-MALAT1 treatment (**Figure 5C**).

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**Figure 5. MALAT1 down-regulation decreased c-Myc expression in protein level.** The expressions of c-Myc in protein level (A, C) and in mRNA level (B) were determined in bone marrow-EPCs with the treatment of si-MALAT1 (or si-control). \*\* *P*<0.01, vs. si-control.

**Improved glucose tolerance in db/db mice given EPCs treated with pioglitazone**

The therapeutic effect of EPCs treated with pioglitazone and si-MALAT1 on glucose tolerance of db/db mice was investigated. The results showed that EPCs from mice treated with pioglitazone resulted in improvement of glucose tolerance. However, when EPCs treated with pioglitazone and si-MALAT1 were administered, the benefit of pioglitazone for blood glucose control was largely reversed (**Figure 6**).

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**Figure 6. Effects of EPCs treatment on** **glucose tolerance in db/db mice.** The db/db mice were administrated autologous EPCs with the different treatments. Group 1: EPCs without any treatment; Group 2: EPCs with the treatment of pioglitazone; Group 3: EPCs with the treatment of pioglitazone and pLVX-IRES-ZsGreenl (control); Group 4: EPCs with the treatment of pioglitazone and pLVX-IRES-ZsGreenl-si-MALAT1. A glucose tolerance test was then performed in db/bd mice. \*\* *P*<0.01, vs. Group 1; # *P*<0.05, ## *P*<0.01, vs. Group 3.

**Discussion**

In the present study, we found that pioglitazone significantly increased the number and the function of circulating EPCs. When EPCs were pre-incubated with pioglitazone and then administered to db/db diabetic mice, a significant decrease in blood glucose level and improvement of glucose tolerance were observed. What is more, up-regulation of MALTA1 expression induced by pioglitazone was proposed to take part in the increasing number and function of EPCs and to provide benefit for the treatment of T2DM.

EPCs co-express surface markers of both hematopoietic stem cells (CD34 and CD133) and endothelial cells (KDR) [23]. The measurement of these three cell markers is commonly used to determine the number of EPCs. In agreement with previous observations, we demonstrated that T2DM patients had a reduced number and function of circulating EPCs [5, 24]. During the last two decades, emerging studies have illustrated that circulating EPCs act as a biomarker of endothelial function and their alteration is characterized by a high risk of cardiovascular disease [25-27]. The impairment and reduction of EPCs are also hallmark features of type 1 and type 2 diabetes [28-30]. Autologous EPCs are considered to be a novel cellular therapy for diabetic vascular complications [31]. However, the application is limited in diabetic individuals due to their dysfunctional circulating EPCs. Previous studies confirmed that pioglitazone increases the number and function of EPCs in individuals with diabetes mellitus [32], coronary artery disease, normal glucose tolerance [33] and impaired glucose tolerance [10], contributing to the better use of EPCs in cardiovascular disease treatment. However, few studies have been shown to investigate the molecular mechanism of pioglitazone on EPCs regulation.

LncRNAs are implicated in diverse human diseases, especially in cancer, cardiovascular disorders and immunological diseases [34]. MALAT1 is first reported in lung cancer and associated with metastasis, migration, tumor growth and cell proliferation in various cancers [16, 35, 36]. Recently, Michalik et al reported that MALTA1 indirectly triggers cell cycle blockade via the up-regulation of cell-cycle-related factors and enhances migration capacity by so-far-unknown mechanisms, and the deficiency of MALAT1 leads to the lower endothelial cell numbers and angiogenic defects [37]. It has also been reported that circulating MALAT1 expression was higher in acute myocardial infarction patients than that in healthy volunteers [38]. Poller et al showed that MALAT1 participates in the process of cardiac innate immunity [39]. In the current study, a decreased MALAT1 expression was observed in circulating EPCs of T2DM patients. Its expression was also reduced in bone marrow-EPCs exposed to high glucose in a dose-dependent manner. Considering the reduced number and function of circulating EPCs, we proposed that MALAT1 was correlated with EPCs function in T2DM patients. As expected, pioglitazone treatment contributed to the elevation of MALAT1 level in EPCs both *in vivo* and *in vitro*.

c-Myc is a regulator gene that codes for a transcription factor and plays a critical role in cell growth, apoptosis, and metabolism in mammals [40]. It has been commonly considered to be a proto-oncogene and is vital for adequate vascular development and angiogenesis [22]. The dysregulation of c-Myc expression is closely related to cancers and cardiovascular disorders [41]. Florea et al demonstrated that the loss of c-Myc expression in endothelial cells results in a pro-inflammatory senescent phenotype, suggesting a central regulator of endothelial dysfunction [21]. In the present study, T2DM patients had decreased c-Myc expression of circulating EPCs in mRNA and protein levels. Pioglitazone treatment up-regulated these expressions of EPCs exposed to high glucose conditions in both *in vivo* and *in vitro*. Previous studies have shown that c-Myc expression is essential for transcriptional repression of p21 and p15 [42, 43]. Therefore, decreased c-Myc levels in circulating endothelial cells may lift repression of these cell cycle inhibitors triggering cell cycle arrest. In transfected bone marrow-EPCs with si-MALAT1 for down-regulating its expression, c-Myc expression was reduced only in protein level. In addition, si-MALAT1 largely canceled the role of pioglitazone in increasing c-Myc protein expression of bone marrow-EPCs exposed to high glucose. These data indicate that MALAT1 is involved in the effects of pioglitazone on EPCs function by regulating c-Myc expression.

The main finding of this study was that MALAT1 is down-regulated in circulating EPCs of T2DM patients, and decreased MALAT1 disrupts glucose tolerance. We also observed that pioglitazone is beneficial for increasing the number and function of EPCs and controlling blood glucose, and MALAT1 down-regulation greatly alters the effects of pioglitazone through modulating c-Myc expression. These findings suggest that autologous EPCs with pioglitazone administration hold great promise in the treatment of diabetes mellitus and diabetic vascular complications. MALAT1 may act as a new molecular target to modulate the function of EPCs.

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